# A role for the extraembryonic yolk syncytial layer in patterning the zebrafish embryo suggested by properties of the *hex* gene

Chi-Yip Ho*, Corinne Houart†, Steve W. Wilson† and Didier Y.R. Stainier*

**Recent studies in mouse suggest that the extraembryonic endoderm has an important role in early embryonic patterning [1]. To analyze whether similar mechanisms operate in other vertebrates, we cloned the zebrafish homologue of *Hex*, a homeobox gene that is expressed asymmetrically in the mouse visceral endoderm [2]. Early expression of zebrafish *hex* is restricted to the dorsal portion of the yolk syncytial layer (YSL), an extraembryonic tissue. By the onset of gastrulation, *hex* is expressed in the entire dorsal half of the YSL, which directly underlies the cells fated to form the neural plate. We show that *hex* expression is initially regulated by the maternal Wnt pathway and later by a Bmp-mediated pathway. Overexpression experiments of wild-type and chimeric Hex constructs indicate that Hex functions as a transcriptional repressor and its overexpression led to the downregulation of *bmp2b* and *wnt8* expression and the expansion of *chordin* expression. These findings provide further evidence that the zebrafish YSL is the functional equivalent of the mouse visceral endoderm and that extraembryonic structures may regulate early embryonic patterning in many vertebrates.**

Addresses: *Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, California 94143-0448, USA. †Department of Anatomy and Developmental Biology, University College London, London WC1E 6BT, UK.

Correspondence: Didier Y.R. Stainier
E-mail: didier_stainier@biochem.ucsf.edu

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## Results and discussion

We isolated zebrafish *hex* by screening an embryonic cDNA library using the mouse *Hex* cDNA [3] as a probe. High sequence identity is found among all *Hex* homologs, especially in the highly conserved homeodomain as well as in an amino-terminal proline-rich region (see Supplementary material). This region also contains a conserved 10 amino acid sequence that is related to the TN domain [4] of several other homeobox genes and has been implicated in repressor function [5].

Zygotic hex expression is first detected at the sphere stage (late blastula) in the dorsal-most portion of the YSL. The YSL, an extraembryonic structure unique to teleosts [6], arises from marginal blastomeres which, during the early blastula stage, collapse onto the yolk cell to form a narrow ring around the blastodisc edge [7]. It then rapidly spreads underneath the blastodisc to form, by the dome stage, a complete 'internal' syncytium that persists throughout embryogenesis. Although initially restricted to the marginal region of the dorsal YSL (Figure 1a,b), *hex* expression extends anteriorly to reach the animal pole by the onset of gastrulation (50% epiboly, Figure 1c). Expression of *hex* remains restricted to the dorsal half of the YSL until the end of gastrulation (Figure 1d–f). Other genes have been found to be expressed in the YSL: some, like ApoE, are expressed throughout the YSL [8], whereas others, like the *nodal*-related gene *squint* [9–11] and the homeobox genes *bozozok* [12–14] and *mixer* [15], are restricted to the marginal region of the dorsal YSL. The expression pattern exhibited by *hex* is different, as although it initiates in the dorsal marginal region of the dorsal YSL like the expression of *squint*, *bozozok* and *mixer*, it then spreads to cover the entire dorsal half of the YSL and underlie the cells fated to form the neural plate [16].

As early as 1936, Oppenheimer proposed that the yolk cell provides developmental cues necessary for teleost embryo formation [17]. Further studies [12–14,18–21] have led to the hypothesis that the YSL functions as the fish equivalent of the Nieuwkoop center, and that the translocation of activated maternal determinants into the dorsal YSL leads to the formation of an 'organizer-inducing center'. This model predicts the existence of genes, such as *hex*, that are specifically activated in the dorsal YSL. To learn more about the regulation of *hex* activation, we examined whether manipulation of the maternal Wnt pathway, which is required for Nieuwkoop center formation and dorsal organizer induction [22], affects *hex* expression. We found that LiCl treatment, which leads to the formation of radially symmetrical dorsoanteriorized embryos [23], caused *hex* expression to spread throughout the YSL (see Supplementary material), and that overexpression throughout the embryo of *β-catenin*, an effector of the Wnt signaling pathway, led to ectopic activation of *hex* specifically in the YSL (Figure 1g,h). These data indicate that *hex* lies downstream of the maternal Wnt pathway and that the YSL is uniquely competent to express *hex* at early gastrulation stages. Recent experiments in *Xenopus* have also shown that *Xhex* expression is regulated in part by the maternal Wnt pathway [24].

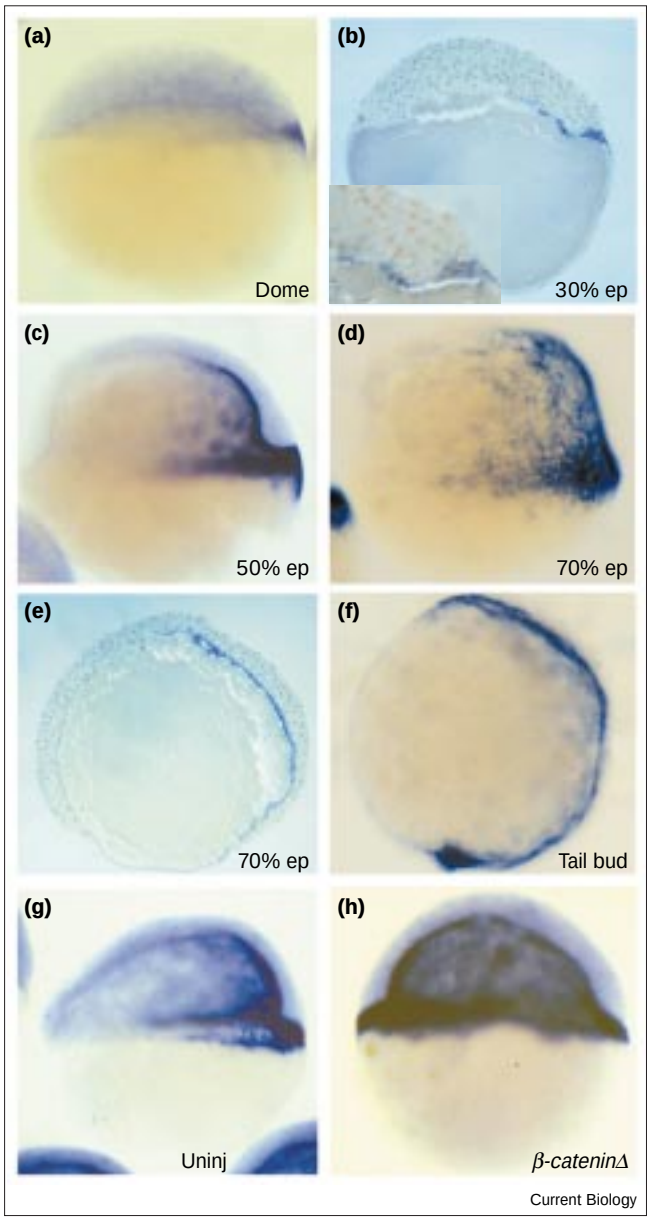



**Figure 1**

Zebrafish *hex* expression is restricted to the dorsal YSL from pre-gastrula stages until the end of gastrulation. **(a–f)** Expression of *hex* is shown at various embryonic stages as indicated; ep, epiboly. Lateral views are shown, with animal pole on top and dorsal to the right. (b,e) The localization of *hex* transcripts was confirmed by sectioning the whole-mount *in situ* hybridized embryos. The inset in (b) shows a higher magnification view of the dorsal side. The photograph in (d) is focused on the YSL. (c) By 50% epiboly (the onset of gastrulation), *hex* expression has almost reached the animal pole. (f) By the end of gastrulation, *hex* expression in the YSL is observed on the entire dorsal half of the embryo from the anterior (top) to the posterior (bottom). Expression of *hex* is also seen at postgastrulation stages in angioblasts and in the liver and thyroid primordia (our unpublished observations). **(g,h)** Expression of *hex* in 50% epiboly embryos is affected by manipulating the maternal Wnt pathway. (g) Uninjected embryos show only dorsal *hex* expression. (h) When *β-catenin* (data not shown) or *β-cateninΔ* are overexpressed throughout the embryo, ectopic *hex* expression is detected in the lateral and ventral YSL. Note that *hex* expression appears to be restricted to the YSL even when *β-catenin* is overexpressed throughout the embryo.

The widespread expression of *hex* on the dorsal side of the YSL throughout gastrulation suggests that this gene is involved in regulating dorsoventral (DV) patterning. To address this possibility, we overexpressed *hex* and examined the expression of *bmp2b* [25,26] and *chordin* [27–29], two genes that are required to establish the gradient of Bmp signaling essential for DV patterning. Overexpression of *hex* led to a profound reduction in *bmp2b* expression (Figure 2a,b) and to an expansion of *chordin* expression ventrally (Figure 2c,d). Consistent with this loss of *bmp2b* and gain of *chordin* expression, we observed an expansion of anterior neural tissue as revealed by *otx2* [30] expression (Figure 2e,f and see Supplementary material). These effects were observed when *hex* was overexpressed throughout the embryo as well as specifically in the YSL. These observations suggest that Hex activity in the dorsal YSL influences patterning of overlying embryonic tissues during gastrulation.

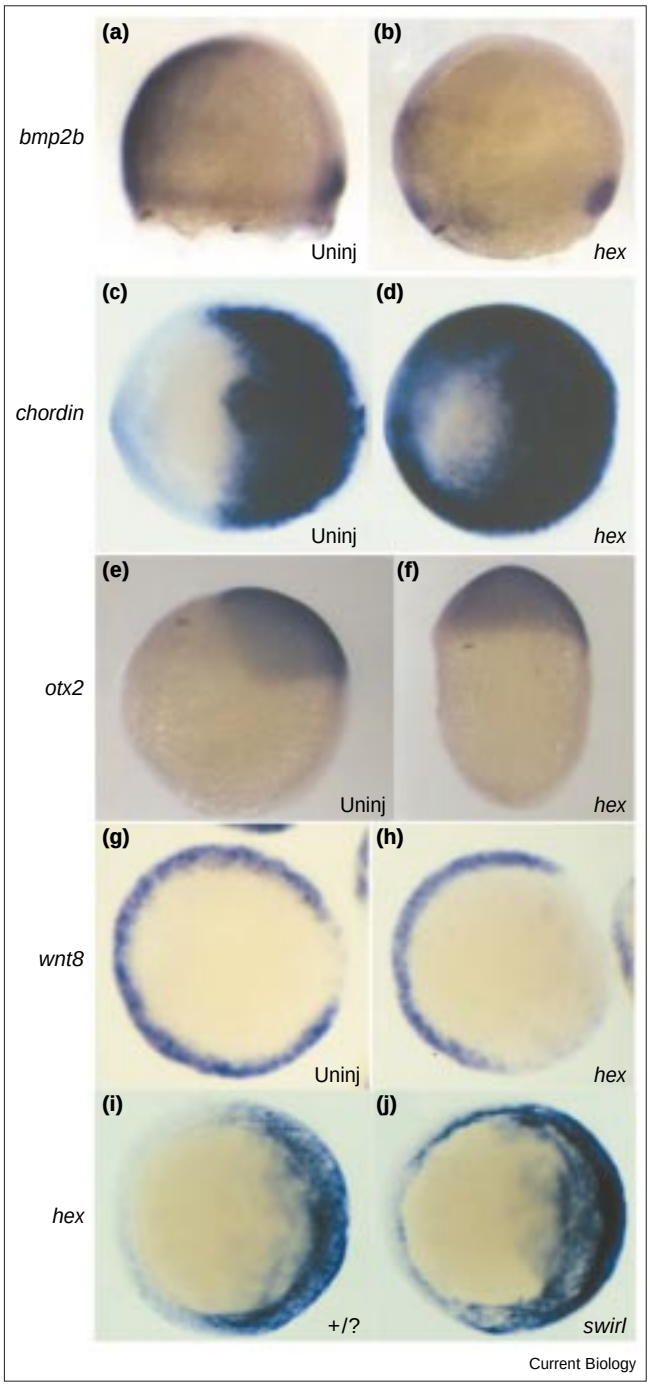
In contrast to *bozozok* and *squint*, which can induce radial expression of *goosecoid* and duplicated axes when overexpressed [9–11,12–14], *hex* overexpression did not lead to duplicated axial structures. In addition, whereas *bozozok* expression is restricted to the marginal region of the dorsal YSL and disappears by the onset of gastrulation, *hex* expression spreads all the way to the animal pole and is maintained throughout gastrulation. These data suggest that the YSL plays both an early role associated with Nieuwkoop center activity and a later, continued role in regulating gene expression on the dorsal side of the embryo throughout gastrulation.

The zygotic Wnt signaling pathway has been implicated in both DV and anteroposterior patterning of the embryo. We found that overexpression of *hex* in the YSL led to a partial loss of *wnt8* [31] expression (Figure 2g,h and see Supplementary material). This reduction was observed as early as 30% epiboly, in other words at a stage before significant loss of *bmp2b* or gain of *chordin* expression, raising the possibility that the loss of *wnt8* expression is independent of Bmp signalling. In support of this model, we observed that *wnt8* expression appears to be unaffected in *swirl/bmp2b* [25,26] and *dino/chordin* [27–29] mutants, at least until the onset of gastrulation (data not shown). We also noted that, at 30% epiboly, *wnt8* and *hex* appear to be expressed in mutually exclusive domains in the YSL (see Supplementary material). Altogether, these data show that Hex can function to downregulate the expression of *bmp2b* and *wnt8*.

As both *bmp2b* [25,32] and *wnt8* are expressed in the YSL, we hypothesized that Hex could directly suppress their expression if it functioned as a transcriptional repressor. To test whether Hex has repressor function, we constructed

**Figure 2**

Overexpression of *hex* causes altered expression of DV patterning genes. Expression of (a,b) *bmp2b*, (c,d) *chordin*, (e,f) *otx2* and (g,h) *wnt8* was detected by *in situ* hybridization in (a,c,e,g) untreated embryos (uninj) and (b,d,f,h) embryos overexpressing *hex* in only the YSL (see Materials and methods). (a,b,e,f) Lateral views with animal pole on top and dorsal to the right; (c,d,g,h) animal pole views with dorsal to the right.
(a,b) Overexpression of *hex* in the YSL reduces ventral *bmp2b* expression in both the YSL and blastoderm at 75% epiboly, but dorsal *bmp2b* expression in the involuting mesendoderm is unaffected and similar to that of the control embryo. (d) Expression of *chordin* is significantly expanded in *hex*-injected embryos at 70% epiboly. (f) Anterior neural tissue, as marked by *otx2* expression, is also increased as a result of overexpressing *hex* in the YSL; note that this 80% epiboly embryo was injected with a slightly higher dose of *hex* mRNA, leading to the elongated shape characteristic of a dorsalized phenotype. (g,h) Overexpression of *hex* in the YSL leads to a partial loss of *wnt8* expression at 40% epiboly. Overexpressing *hex* throughout the embryo can cause near complete loss of *wnt8* expression as well as premature *otx2* expression (see Supplementary material). (i,j) In *swirl* mutants, Bmp2b is inactive and ventrally expanded expression of *hex* is observed at 70% epiboly relative to heterozygous or wild-type siblings (+/?).




chimeric proteins consisting of the engrailed repressor domain linked to the carboxy-terminal 116 amino acids of Hex containing the homeodomain (En–HD) or to full-length Hex (En–Hex). Injection of mRNAs encoding these proteins showed that En–Hex (see Supplementary material) and En–HD (data not shown) had similar effects to those of wild-type Hex in modulating the expression of *bmp2b*, *chordin*, *otx2* and *wnt8*. These data suggest that zebrafish Hex normally functions as a transcriptional repressor, in agreement with recent functional characterization of rat Hex [33].

Because of the observed effect of *hex* overexpression on *bmp2b* expression, we also examined *hex* expression in *swirl*/*bmp2b* and *dino*/*chordin* mutants [25–29]. We found that, in *swirl* mutants, although expression is initially normal, there is a gradual ventral expansion of *hex* expression such that by the end of gastrulation *hex* is expressed throughout the YSL (Figure 2i,j). Conversely, *chordin* mutants show a gradual reduction of *hex* expression during gastrulation (see Supplementary material). Recent work in *Xenopus* has shown that Bmp4 downregulates *Xhex* expression [24]. These data indicate that *hex* and *bmp2b* can negatively regulate each other and that a complex network of interactions ensures their proper spatial expression during gastrulation.

Experimental evidence in a number of systems indicates that *bmp* gene expression is controlled by an autoregulatory feedback mechanism and that *chordin* expression is negatively regulated by Bmp signaling ([25–29], reviewed in [34]). Chordin can, in turn, negatively regulate *bmp* expression, presumably by inhibiting Bmp activity and interrupting the autoregulatory feedback loop. In zebrafish, one possibility is that *bmp2b* is initially activated in the YSL and that this expression leads to *bmp2b* activation in the overlying cells of the blastoderm. The expression of *hex* and *chordin* on the dorsal side of the embryo may then lead to the repression of *bmp2b* in both the dorsal YSL and the overlying cells of the blastoderm. Involvement of Hex in other early developmental pathways is of course possible, especially since Hex also affects *wnt8* expression and since the mechanisms by which the Wnt and Bmp pathways interact during DV and AP patterning are, as yet, largely unknown.

The continuous expression of *hex* in the dorsal YSL throughout gastrulation suggests that this extraembryonic

tissue plays an important role not only in the induction of the dorsal organizer but also at later stages of embryogenesis. We propose that *hex* expression in the YSL acts to regulate *bmp2b* and *wnt8* expression in the embryo. Analogous to the proposed function of Hex in zebrafish, *hex* expression in the mouse embryo may also serve to inhibit the expression of *Bmp* genes in the AVE and, through cell non-autonomous mechanisms, in the anterior part of the embryo proper [1]. Furthermore, both the dorsal YSL and the AVE are in close apposition with the neural ectoderm before the mesendoderm migrates under it during gastrulation [1,35]. The dorsal YSL and AVE may thus also contribute to the induction and/or patterning of anterior neural tissue by inhibiting both the Bmp and Wnt pathways [36]. Our studies, together with those of others [14,35], emphasize that the YSL, much like the mouse visceral endoderm, is finely patterned and that it may have additional roles besides functioning as a Nieuwkoop center in patterning the body axes during early embryonic development.

*Supplementary material*

Supplementary material including additional data and methodological detail is available at http://current-biology.com/supmat/supmatin.htm.

## Acknowledgements

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# Supplementary material

# A role for the extraembryonic yolk syncytial layer in patterning the zebrafish embryo suggested by properties of the *hex* gene

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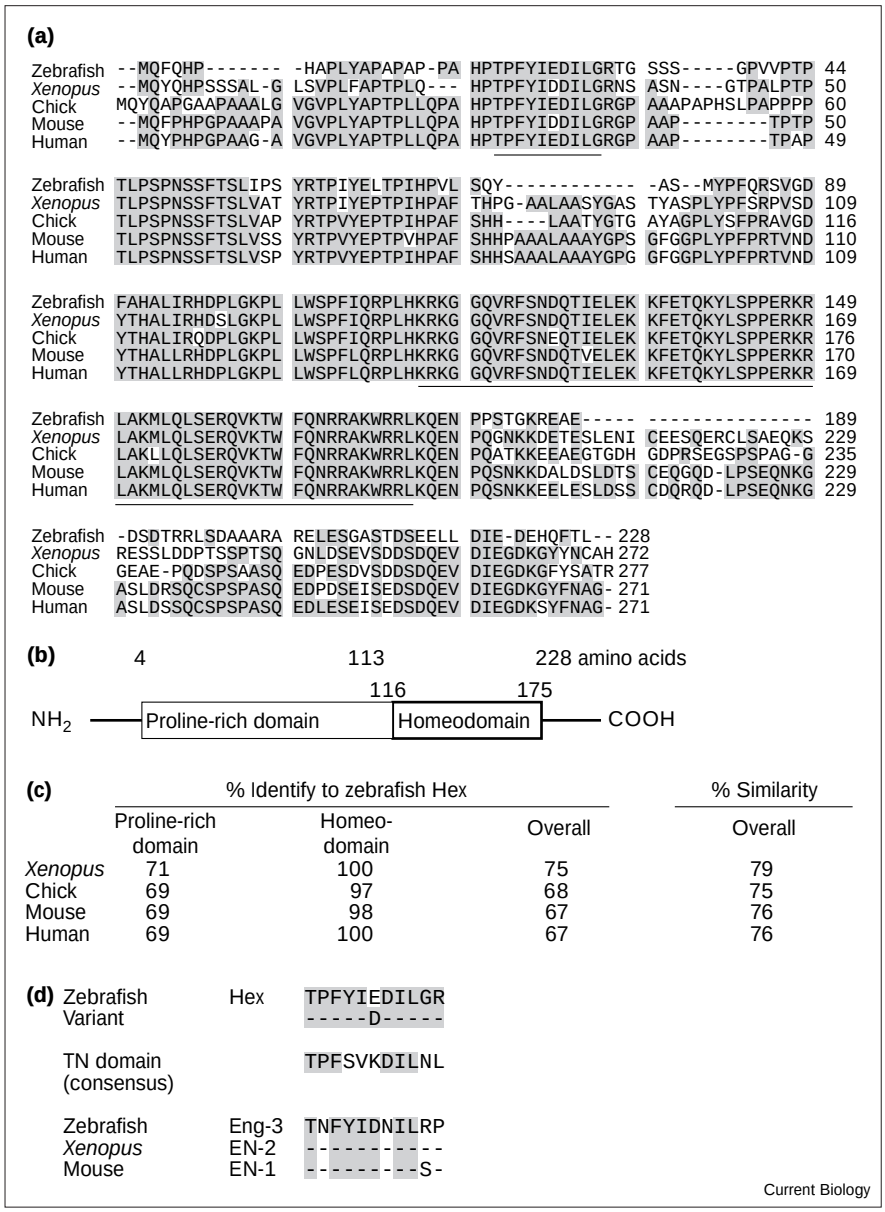
**Figure S1**

Amino acid sequence comparison of vertebrate Hex proteins. Zebrafish Hex shares high sequence identity with its homologs *Xenopus* Xhex [S3], chick Prh (proline-rich homeobox) [S4], mouse Hex and human HEX [S5]. The zebrafish Hex amino acid sequence shown is deduced from the cDNA isolated (GenBank accession number AF131070). Dashes represent gaps, and residues that are identical in all or most homologs are shaded. Sequence analysis indicates remarkably high sequence identity in two distinct domains: the proline-rich domain (residues 4–113) and the DNA-binding homeodomain (residues 116–175, underlined). The TN-like sequence that is similar to the short sequence essential for the repressor function of engrailed is also underlined (residues 22–31). **(b)** Schematic representation of the domains of zebrafish Hex. **(c)** Percentage identities and similarities of zebrafish Hex with its homologs. **(d)** An alignment of TN-like sequences; dashes indicate identical residues and 'variant' shows the only change that is present in the *Xenopus* and mouse proteins – see (a).




## Supplementary materials and methods

### *Cloning of* hex

A λgt11 cDNA library from 33–36 h zebrafish embryos was obtained from K. Zinn and screened using standard techniques.

### In situ *hybridization and histology*

For wholemount *in situ* hybridization, embryos were fixed in 4% paraformaldehyde in PBS at various stages. Expression of *hex*, *bmp2*, *wnt8*, *chordin* and *otx2* was detected using digoxigenin-labeled antisense RNA probes. For histology, whole-mount stained embryos were embedded in methacrylate JB4 resin and cut into 10 μm sections, and the sections counterstained with Neutral Red.

### *Embryonic manipulations and microinjection experiments*

LiCl (0.3 M) was applied to 32-cell-stage embryos for 10 min to induce radial dorsalization as described [S1]. For overexpression throughout

**Table S1**

**Overexpression of *hex* causes altered expression of DV patterning genes.**

| Hybridization probe | Stage of embryo examined | RNA injected into YSL | Total number | Number of embryos showing: Normal expression | Reduced expression | Extended expression |
|---|---|---|---|---|---|---|
| *bmp2b* | 60–90% epiboly | *hex* | 621 | 348 | 272 | 1 |
| *bmp2b* | 60–90% epiboly | *en–hex* | 330 | 176 | 154 | 0 |
| *bmp2b* | 60–80% epiboly | *lacZ* | 179 | 170 | 5 | 4 |
| *chordin* | 60–80% epiboly | *hex* | 320 | 122 | 0 | 198 |
| *chordin* | 60–80% epiboly | *en–hex* | 129 | 53 | 0 | 76 |
| *chordin* | 60–80% epiboly | *lacZ* | 178 | 174 | 0 | 4 |
| *otx2* | 60–85% epiboly | *hex* | 134 | 51 | 0 | 83 |
| *otx2* | 60–85% epiboly | *en–hex* | 68 | 25 | 0 | 43 |
| *otx2* | 60–85% epiboly | *lacZ* | 55 | 54 | 0 | 1 |
| *wnt8* | 30–50% epiboly | *hex* | 150 | 111 | 39 | 0 |
| *wnt8* | 30–50% epiboly | *en–hex* | 98 | 75 | 23 | 0 |
| *wnt8* | 30% epiboly | *lacZ* | 20 | 20 | 0 | 0 |

the embryo, 5′-capped mRNAs were transcribed from pCS2(+) derivatives of zebrafish *hex*, zebrafish *β-catenin* or *Xenopus β-catenin*Δ, an amino-terminally truncated constitutively active version [S2], and approximately 100 pg of the synthetic transcript was injected into the yolk of 1–4-cell stage embryos. As a control, we also used a similar concentration of RNA from a glutathione-S-transferase–green-fluorescent-protein construct (GST–GFP). To restrict overexpression to the YSL, we injected mRNA (100 pg) into the yolk at about the 1000-cell stage, as injection of *lacZ* or *GFP* mRNA after the 256-cell stage leads to β-galactosidase or GFP expression confined to and spread throughout the YSL (Jeremy Reiter, C.H., S.W.W. and D.Y.R.S., unpublished observations). Injection of a control mRNA, such as *lacZ*, into the YSL caused the expression of *bmp2*, *chordin*, *otx2* and *wnt8* to be affected in 0–4% of the embryos (Table S1). A pCS2(+) derivative containing the Engrailed effector domain was used to construct the En–Hex fusions. Full-length *hex* cDNA or the carboxy-terminal half (HD, amino acids 113–228, containing the homeodomain) were PCR amplified from pCS2(+)*hex*, subcloned into the 3′ *Xho*I–*Xba*I site of the pCS2(+)-En plasmid and sequenced to check amplification accuracy.

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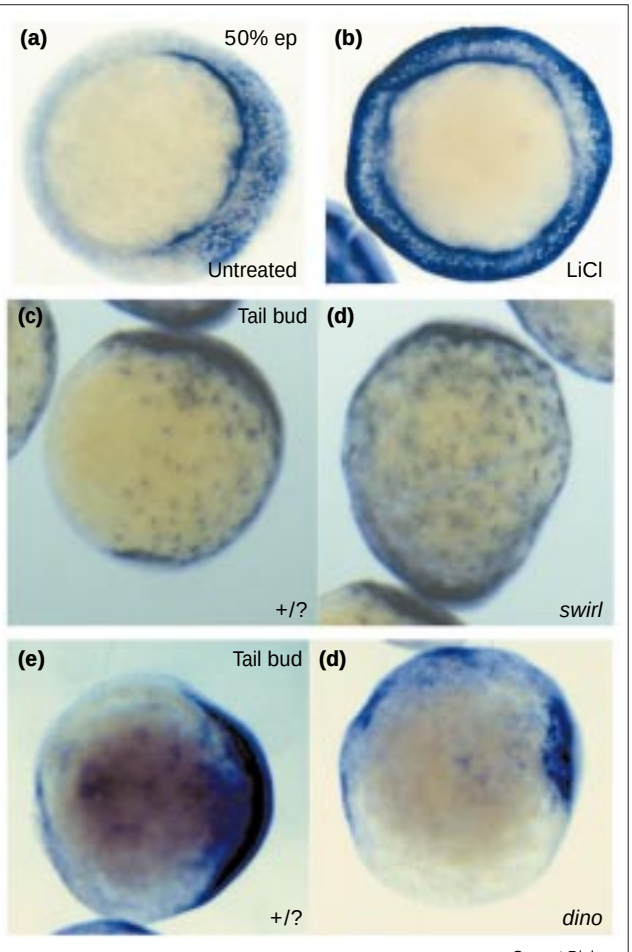
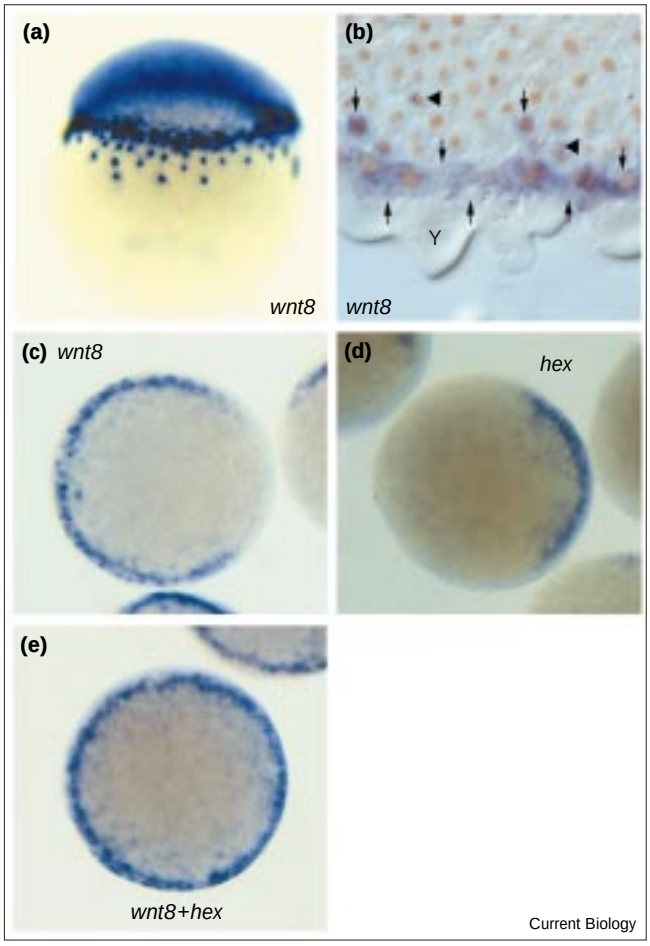
**Figure S2**



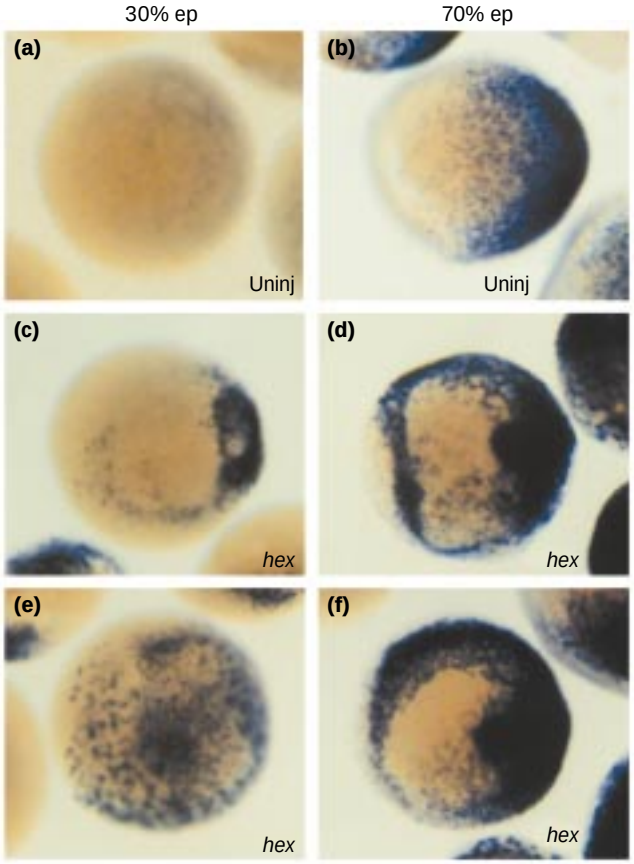

Expression of *hex* is affected by manipulation of the maternal Wnt pathway as well as by Bmp2b and Chordin. (**a**,**b**) Animal pole views of 50% epiboly embryos, dorsal to the right. Whereas the uninjected embryos (a) show only dorsal *hex* expression, exposure to LiCl at the 32-cell stage (b), a treatment known to generate an overall dorsalizing effect through the maternal Wnt pathway, radializes *hex* expression in the YSL. (**c**,**d**) In *swirl* mutants, Bmp2b is inactive and ventrally expanded expression of *hex* relative to heterozygous or wild-type siblings (+/?) is observed. A lateral view at tail-bud stage, animal pole on top and dorsal to the right, is shown. (**e**,**f**) In *chordin* mutants, a reduction of *hex* expression is seen. An anterior view at tail-bud stage, dorsal to the right, is shown.

**Figure S3**



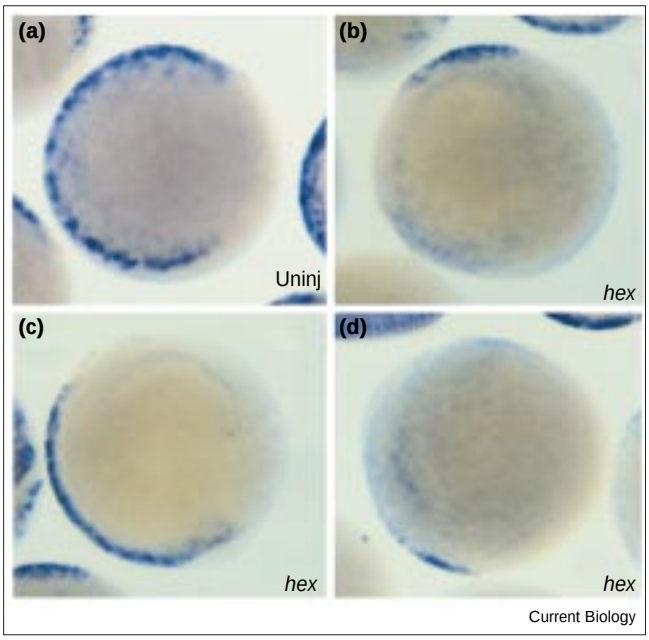

The *wnt8* gene is expressed in the YSL and *hex* and *wnt8* expression patterns appear complementary in the late blastula. (**a**) Ventrolateral view of a 30% epiboly embryo stained for *wnt8* expression by *in situ* hybridization, animal pole on top. This embryo was overstained to reveal the expression of *wnt8* in the external YSL. (**b**) Histological observations of the same embryo confirm the presence of *wnt8* transcripts around the large nuclei of the YSL (arrows) and of a few marginal blastomeres (arrowheads). (**c–e**) Animal pole views of embryos stained for expression of (c) *wnt8*, (d) *hex* and (e) both at 30% epiboly, dorsal to the right. At this stage, *hex* and *wnt8* expression domains in the YSL appear complementary. Y, yolk.

**Figure S4**




Overexpression of *hex* leads to premature and ectopic *otx2* expression. Animal pole views of uninjected embryos (uninj) and embryos overexpressing *hex* throughout (*hex*). (a) The normal onset of *otx2* expression is at 60% epiboly, but (c,e) *hex* overexpression throughout the embryo leads to premature *otx2* expression as examined at 30% epiboly (52%, *n* = 92). (e) A small but significant number of embryos (5%, *n* = 92) have evenly distributed patterns of *otx2* expression, which do not resemble the normal pattern. (d,f) When examined at 70% epiboly, a large number of *hex*-injected embryos (47%, *n* = 147) display ectopic *otx2* expression in addition to a main patch of staining that resembles the normal pattern. These ectopic sites of *otx2* expression are found mostly in the lateral and ventral marginal zones and less frequently extend ventrally from the animal pole.

**Figure S5**




Overexpression of *hex* leads to a significant loss of *wnt8* expression. Expression of *wnt8* in (a) control (uninj) and (b–d) injected (*hex*) embryos at 30% epiboly is shown in animal pole views with dorsal to the right. When *hex* is overexpressed throughout the embryo, significant loss of *wnt8* expression is observed in most embryos (87%, *n* = 117).